Environmental Health Perspectives
Vol. 93, 111–119, 1991

Gene Regulation and Genetic Susceptibility to Neoplastic Transformation: AP-1 and p80 Expression in JB6 Cells

by Lori R. Bernstein,* Elia T. Ben-Ari,* Stephanie L. Simek,*† and Nancy H. Colburn*

The mouse epidermal JB6 cell system consists of clonal genetic variants that are sensitive (P^+) or resistant (P^-) to the promotion of neoplastic transformation by phorbol esters and other tumor-promoting agents. P^+ cells display AP-1-dependent phorbol-ester-inducible transactivation of gene expression, whereas P^- cells have a defect in transactivation. Transfection of promotion sensitivity gene *pro*-1 into P^- cells reconstituted both P^+ phenotype and AP-1-dependent phorbol-ester-inducible transactivation. P^- and P^+ cells exhibited induction of c-*jun* and c-*fos* messenger RNA levels by phorbol ester, but P^- cells had significantly lower basal and induced levels of *jun* mRNA than P^+ cells. Basal and induced levels of c-*jun* protein were significantly lower in P^- cells as well. Differences in levels the 80-kDa pI 4.5 protein p80 were also observed in JB6 cells as a function of preneoplastic progression; high levels of p80 protein and mRNA were observed in P^- cells, intermediate levels in P^+ cells, and negligible levels were observed in transformed derivatives of JB6 cells. Phorbol ester treatment induced phosphorylation but not synthesis of p80. These data are consistent with the hypotheses that AP-1 is required in the signal transduction pathway for promotion of neoplastic transformation by tumor promoter, that *pro* genes may control AP-1 activity, that threshold levels of Jun mRNA and protein may play a role in transactivation and promotion sensitivity, and that the p80 protein in JB6 cells may behave *in vivo* as a suppressor of cellular transformation.

Introduction

Recent progress in understanding the genetics of susceptibility to tumor promotion has come from *in vivo* studies in the mouse by Drinkwater (*1*), DiGiovanni (*2*), Gould et al. (*3*), and Malkinson (*4*) and from studies with mouse epidermal JB6 cell variants in our laboratory and those of others. The JB6 cell lines were derived from untreated primary BALB/c mouse epidermal cell cultures that gave rise at a very low frequency to immortalized cell lines (*5*). The immortalized JB6 cells underwent further change to stably acquire sensitivity to induction of anchorage independence and tumorigenicity by phorbol esters such as tetradecanoyl phorbol-13-acetate (TPA) and other tumor promoters (*5,6*). Nonselective cloning soon after observation of this change yielded clonal lines that were stably sensitive (P^+) or resistant (P^-) to tumor-promoter-induced neoplastic transformation (*7–9*). The percentage of cells in agar that display TPA-induced anchorage independence is typically in the range of 20% for P^+ cells and 0.2% or less for P^- cells. These JB6 variants sensitive to tumor-promoter-induced transformation appear to undergo a transition analogous to second-stage tumor promotion *in vivo* since second-stage tumor promoters such as mezerein induce transformation, and second-stage inhibitors, such as retinoids, but not first-stage inhibitors, such as antiproteases, block the induced transformation (*10*).

Table 1 summarizes the results of a number of studies that have used the P^+ and P^- variants to identify steps that may be required in the signal transduction pathway for promoter-induced transformation. Candidates for required events would be expected to show a P^+/P^- differential in some or all clonal variants tested; responses not showing a P^+/P^- differential may or may not be required ones. P^+ and P^- cells showed similar responses to mitogenic stimulation by phorbol esters, and displayed similar induction of protease activity. They also showed similar levels of protein kinase C activity. Whether there are protein kinase C subtype differences has not yet been established.

*Cell Biology Section, Laboratory of Viral Carcinogenesis, National Cancer Institute-FCRDC, Frederick, MD 21702.

†Present address: Biological Carcinogenesis and Development Program, Program Resources, Inc., NCI-FCRDC, Frederick, MD 21702.

Address reprint requests to L. R. Bernstein, Cell Biology Section, Laboratory of Viral Carcinogenesis, National Cancer Institute-FCRDC, Frederick, MD 21702.

Table 1. Biochemical and genetic responses to tumor promoters in P^+ and P^- cells.

P^+ and P^- cells show the following similar responses to tumor promoters:
Mitogenic stimulation from quiescence (*11*)
Decreased synthesis of collagen, fibronectin (*21–23*)
Induction of proteases: major excreted protein and plasminogen activator (N. H. Colburn and K. Hirano, unpublished)
Increased glucose uptake and ornithine decarboxylase activity (*24,25*)
Similar protein kinase C activation and substrates (*10,12,13*)
What distinguishes P^+ from P^- cells?
Activated versus inactive *pro* 1 and *pro* 2 (*14,15,26*)
Levels of an 80-kDa/pI 4.5 phosphoprotein (*10,13,20*)
Ganglioside synthesis response to TPA (*16,27*)
Induction of AP-1/*jun*-dependent transactivation of gene expression (*19*) and *jun* mRNA and protein levels
Induction of 15-kDa and 16-kDa nuclear proteins (K. Hirano and N. H. Colburn, in preparation)
DNA damage and poly ADP ribosylation responses (*18*)

JB6 P^+ but not P^- cells possess activated DNA sequences called *pro* 1 and *pro* 2 that confer sensitivity to tumor-promoter-induced transformation when transferred into P^- cells (*14*). *Pro* 1 appears to encode a transcript whose synthesis is catalyzed by RNA polymerase III (*15*). The mode of activation of *pro* 1 to a P^+ active structure is not yet known. P^+/P^- differences in TPA modulated ganglioside synthesis (*16*) and induced synthesis of 15 and 16 kDa nuclear proteins (K. Hirano, B. Smith, and N.H. Colburn, in preparation) have also been observed. Nakamura et al. have reported that treatment of JB6 P^+ cells with xanthine-xanthine oxidase, which generates superoxide anion and subsequently other active oxygen species, promotes neoplastic transformation (*17*). Cerutti and co-workers have found that JB6 P^- cells show a greater DNA damage and ADP ribosylation response to transformation-promoting xanthine-xanthine oxidase treatment than do P^+ cells (*18*). These results suggest that greater oxidant defense may be an important component of the P^+ phenotype. Finally, Table 1 shows two other sets of genes whose expression and/or activity is differential in P^+ and P^- cells. These are the AP-1 transactivating complex composed of members of the *jun* and *fos* multigene families (*19*), and an 80-kDa pI 4.5 protein (p80). The present communication focuses on AP-1 and p80 and their roles in preneoplastic progression in JB6 cells.

AP-1/*jun*-Mediated Transactivation of Gene Expression Is Differentially Induced by Tumor-Promoting Agents in P^+ Cells and P^- Cells

TPA treatment of cells induces the expression of a number of genes, some of which encode proteins thought to be key participants in implementing neoplastic transformation (*28,29*). The list of such TPA-inducible genes includes several proto-oncogenes, including c-*myc* and c-*fos*, proteases, including collagenase, stromelysin and plasminogen activator, numerous virally encoded genes, and other sequences (*28,29*). Investigation of the identities of *trans*-regulatory factors that would be predicted to exist and to control *cis* enhancer elements in the promoter regions of these sequences led to the discovery of AP-1 transactivating protein (*30,31*). The complex consists of a heterodimeric species containing products of the *jun* and *fos* multigene families (*32–35*); homodimeric Jun protein complexes have also been detected (*35*).

TPA induces AP-1 binding to a consensus upstream regulatory enhancer sequence (TGACTCA) in several genes thought to be involved in oncogenesis (*30*). The binding of AP-1 to its enhancer is likely to regulate transcription of these genes. We therefore hypothesized that AP-1 function is specifically required for the *promotion* phase of neoplastic transformation. If AP-1 controls a set of effector genes required for tumor-promoter-induced transformation, then some promotion-resistant variants may owe their resistance to a defect in tumor promoter inducibility of AP-1 function.

To investigate this hypothesis, mouse JB6 P^+ and P^- variants were treated with TPA after transient transfection with plasmid 3XTRE-CAT, a construct that has three tandem TPA-responsive *cis*-enhancer elements attached to the Herpes simplex virus thymidine kinase (HSV-TK) promoter and a gene encoding chloramphenicol acetyltransferase (CAT) (*30*). Induced CAT gene expression in this system depends upon tumor-promoter-mediated activation of cellular AP-1 activity. This system enabled us to test the prediction that cellular genetic variants resistant to the promotion of neoplastic transformation by TPA would possess defective AP-1 transactivation function (*19*).

As shown in Figure 1*A*, P^+ Cl 41 cells displayed significant inducible AP1-dependent CAT protein synthesis within 3.5 hr of TPA treatment (but not within 1.5 hr). TPA-induced expression of CAT reached a maximum of 900 to 1000 units of enzyme activity in the P^+ Cl 41 cells after 48 hr (5- to 6-fold induction) and was persistent over at least 100 hr. In contrast, P^- Cl 30 cells showed little inducibility by TPA at any time point tested from 0 to 100 hr. Uptake of the transfected 3XTRE-CAT plasmid in the R^+ and P^- cells was equalized as measured by Southern hybridization analysis of transient transfectants (*19*) (not shown). Furthermore, equal levels of CAT activity were observed in P^+ Cl 41 cells and P^- Cl 30 cells transfected with the constitutively expressed plasmid pRSVCAT at doses such that DNA uptake (copies per cell) was equalized. AP1-dependent CAT synthesis was also induced in P^+ cells but not in P^- cells by epidermal growth factor (EGF) and by high concentrations of serum, two additional transformation promoting agents for JB6 cells (*19*) (not shown). These data point to a specific regulatory defect at the level of AP-1 function in the promotion resistant Cl 30 cells.

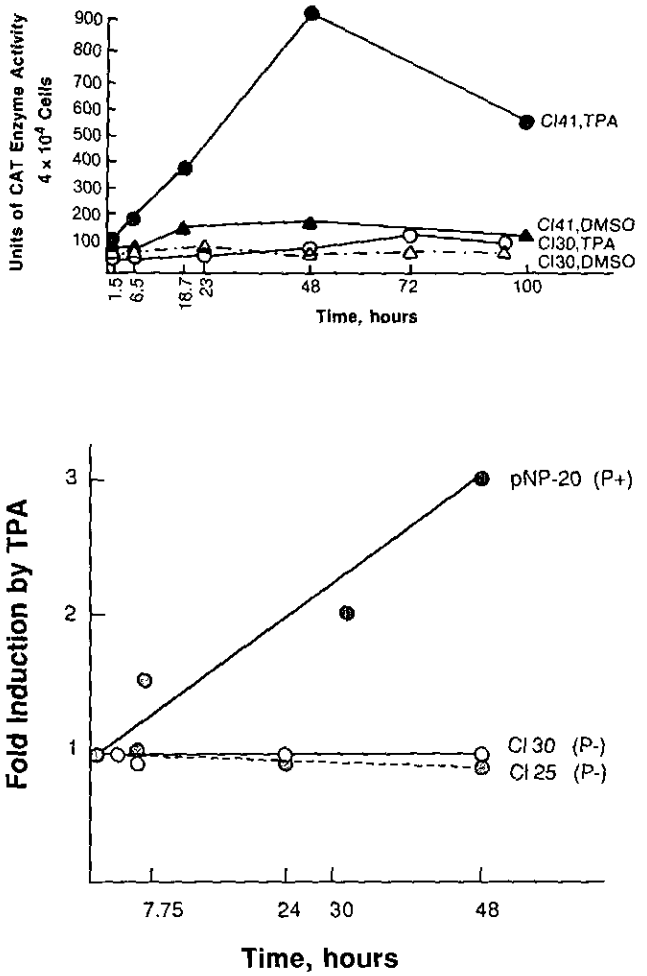

FIGURE 1. TPA induces AP1-dependent CAT synthesis in P^+ cells but not in P^- cells. (*A*) Differential CAT induction in P^+ Cl 41 cells and P^- Cl 30 cells. P^+ Cl 41 cells and P^- Cl 30 cells were plated, TPA treated in 2% fetal calf serum, harvested, and assayed for CAT enzyme activity as described (*19*). TPA and control solvent DMSO treatments were conducted at each time point. Results showing differential inducibility were obtained in three independent experiments. Data from a representative experiment are plotted as units of CAT enzyme activity per 4×10^4 TPA-treated cells divided by the activity for 4×10^4 DMSO-treated cells at that time point. One unit of CAT enzyme activity is defined as activity required to catalyze acetate transfer to chloramphenicol at a rate of 5.4 fmole/min. at 37°C. (*B*) Differential TPA-inducible CAT synthesis in P^+ clonal *pro* 1 transfectants and P^- recipients and Cl 25 cells. Clonal P^+ transfectants, designated pNP cells, were generated as described in the text. Cells were treated and assayed over time courses of TPA treatment as described in *A*, and results are plotted as fold induction versus time of TPA treatment.

Differential AP1-dependent CAT gene regulation in P^+ and P^- cells was also observed in two independently derived clonal JB6 P^+ and P^- cell variants, as is shown in Figure 1*B*. The independent P^- cell line is designated Cl 25, and its time course for AP1-dependent CAT synthesis as a function of TPA treatment reveals nonresponsiveness, as was observed in the P^- Cl 30 cell line. The P^+ clonal variant, designated pNP-20, was derived from parental P^- Cl 30 cells by ring cloning of G418-selected transfectants generated by introduction of a plasmid construct harboring the mouse promotion sensitivity gene *pro* 1 (*28*) and a neomycin resistance marker. This variant displayed anchorage-independent colony formation upon TPA treatment in soft agar (not shown). As shown in Figure 1*B*, this P^+ *pro* 1 transfectant cell line exhibited TPA-inducible CAT gene expression over a 48-hr time course, with a linear increase from 0 to 48 hr. In contrast, the P^- recipient cells did not display induced expression. Note that transfectants harboring the *neo* resistance marker have shown no ability to activate AP-1 function (*36*).

Observation of defective AP-1 function in two independent P^- clonal variants and of competent AP-1 function in two independent P^+ clonal variants demonstrates an association between AP-1 function and promotion of transformation. It is consistent with the hypothesis that AP-1 function is required along the signal transduction pathway for promotion of neoplastic transformation by TPA. Furthermore, the fact that introduction of a gene that confers sensitivity to promotion of transformation by TPA reconstitutes AP-1 function to a defective P^- cell supports the hypothesis that *pro* genes can execute control over the activity of AP-1.

Measurement of *jun* and *fos* mRNA Levels in P^+ and P^- Cells

TPA stimulates the accumulation of c-*jun* mRNA in murine and human fibroblasts (*42,43*) and induces transcription of the c-*fos* gene in a number of systems (*44*). Therefore, the possibility that differential AP-1 dependent transactivation in P^+ and P^- cells was due to differences in expression of c-*jun* or c-*fos* was examined. Total RNA was isolated from JB6 cells at various times after treatment with 10 ng/mL TPA, and the relative levels of c-*jun* or c-*fos* mRNA were determined by Northern blotting and densitometry scanning analyses of the resulting autoradiographs (Fig. 2, upper panel). TPA-induced c-*jun* mRNA levels were higher in P^+ cells than in P^- cells at all times after addition of TPA, and basal levels of this message were also 5- to 10-fold higher in the P^+ cells. In several experiments c-*jun* mRNA was undetectable in untreated P^- cells. After 24 hr, the amount of c-*jun* mRNA returned to basal levels in the P^+ cells, but remained slightly elevated in P^- cells.

In contrast to the results obtained for c-*jun*, TPA-stimulated levels of c-*fos* mRNA were essentially equal in P^+ and P^- cells (Fig. 2, lower panel). Basal c-*fos* mRNA levels were 2.5- to 10-fold higher in the P^- cells. The degree of induction of c-*fos* message by TPA was much greater than that observed for c-*jun*, and the levels of c-*fos* mRNA declined much more

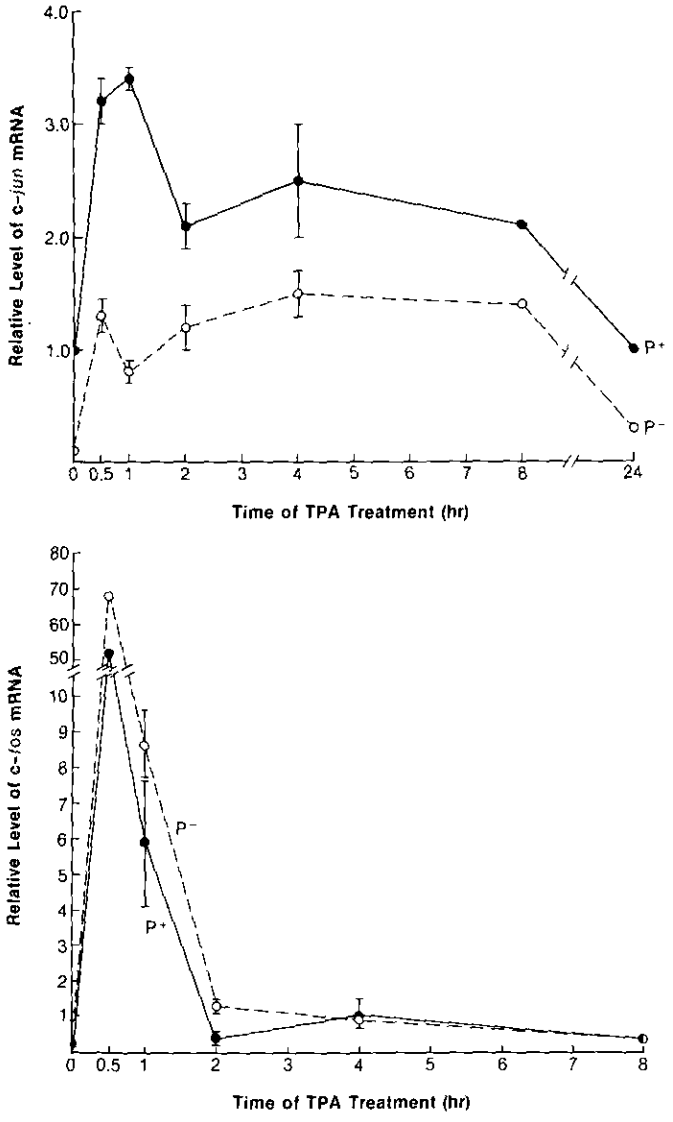

FIGURE 2. TPA differentially induces c-*jun* but not c-*fos* mRNA accumulation in P^+ and P^- cells. Promotion-resistant (P^-) and promotion-sensitive (P^-) JB6 cells were grown to near-confluence in 5% serum in T150 flasks and then switched to 2% serum for 24 hr. The cells were treated with 10 ng/mL TPA (16 nM), harvested by trypsinization and centrifugation, and total RNA was extracted at the indicated times as described previously (*45*). RNA (10 μg/sample) was subjected to electrophoresis and Northern blot analysis (*45*) using v-*jun* or v-*fos* cDNA probes (gifts of P. Vogt and T. Curran, respectively) labeled with ^{32}P by the random priming method (Pharmacia). The relative levels of c-*jun* mRNA (upper panel) and c-*fos* mRNA (lower panel) were determined by densitometric analysis of the resulting autoradiographs. c-*jun* mRNA levels are expressed as values relative to the levels in untreated P^+ Cl 41 cells (i.e., relative *jun* mRNA = 1.0 for Cl 41 at T = 0), and c-*fos* mRNA levels are expressed relative to the values in untreated P^- Cl 30 cells. The data shown are the means of three to four experiments ± SE, except for the following points. For c-*jun*: P^+ value at 0.5 hr (n = 2); 8 hr and 24 hr values (n = 1). For c-*fos*: 0.5 hr values (n = 2); 8 hr value (n = 1).

rapidly than those of c-*jun* mRNA, returning to near-basal amounts after 2 hr of TPA treatment.

The above results suggest that the differential transactivation observed in response to TPA in promotion-sensitive and resistant JB6 cells may be accounted for at least in part by differences in TPA-stimulated accumulation of c-*jun* mRNA. However, differential TPA-induced expression of c-*fos*, at least at the message level, can be ruled out as a contributor to the observed differences in AP-1-dependent transactivation. While AP-1-dependent transactivation via the 3XTRE in response to TPA is virtually undetectable in P^- cells, TPA still stimulates c-*jun* mRNA accumulation to a significant degree in these cells. It is possible that a threshold level of c-*jun* mRNA and protein must be reached in order to stimulate AP-1 dependent transactivation above basal levels.

Expression of Jun Protein in JB6 P^+ and P^- Cells

To determine whether phenotypic differences in c-*jun* mRNA levels are also observable at the protein level, Western immunoblotting analyses of nuclear lysates from TPA-treated P^+ and P^- cells were conducted (*37*) employing antisera specific to the c-*jun* protein. Nuclear proteins from TPA treated cells (10 ng/mL TPA) and untreated cells were run on 10% Laemmli SDS-PAGE gels, transferred onto nitrocellulose filters, and immunoblotted with affinity purified rabbit anti-PEP-2 Jun peptide antiserum (*38*) (Oncogene Science, Manhasset, New York). Anti-PEP-2 specifically recognizes peptide sequence T P T P T Q F L C P K N present in viral Jun, and is crossreactive with mouse c-Jun. After incubation with ^{125}I-protein A, filters were subjected to autoradiography and X-ray films were scanned by densitometry analysis.

Figure 3 shows a representative time course experiment of TPA treatment in P^+ Cl 41 and P^- Cl 30 cells over 24 hr. As was the case for *jun* mRNA, Jun protein was observed at significantly lower levels in the P^- Cl 30 cells than in the P^+ Cl 41 cells at time points examined. Basal levels were approximately 5-fold lower in the P^- cells than in the P^+ cells, and induced levels ranged from 2- to 10-fold lower during the time course. In the P^+ cells, TPA induced a rapid accumulation of Jun protein within 30 min, followed by a decline over the duration of the experiment; in the P^- cells a delay in the onset of induction was observed. In both cell lines Jun protein levels declined by 24 hr of TPA treatment.

These data support the hypothesis that control of Jun protein levels in JB6 P^+ and P^- cells is pretranslational and is most likely caused by differential accumulation of *jun* mRNA. The results provide further support for the hypothesis that differences in transactivation and promotion sensitivity in the P^+ and P^- cells may in part be explained by differences in *jun* mRNA and Jun protein accumulation and are consistent with the possibility that threshold Jun levels are required for transactiva-

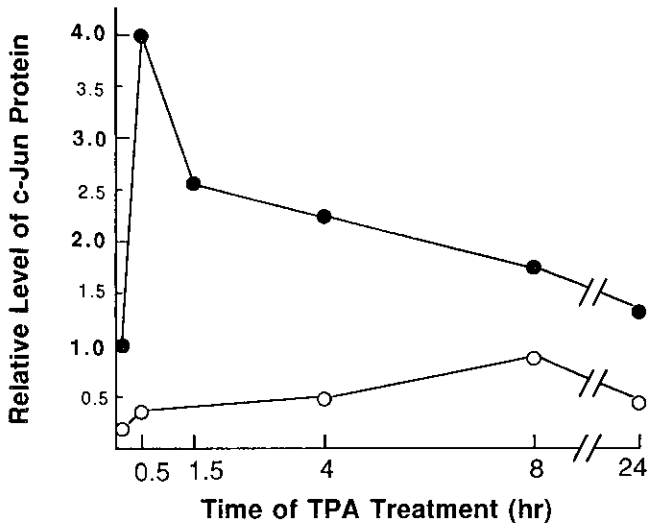

FIGURE 3. Time course of TPA treatment and measurement of c-Jun protein levels in P^+ and P^- Cells. P^+ Cl 41 cells and P^- Cl 30 cells were TPA treated and harvested as described in Figure 2. Nuclei were prepared as described by Bos et al. (*39*) with minor modifications (*37*). Per sample, 1×10^6 nuclei were run in 10% Laemmli SDS polyacrylamide gels (*39,40*), transferred by Western blotting onto BA85 nitrocellulose filters (Schleicher and Schuell), and blotted with 5 μg/mL affinity purified rabbit anti PEP-2 antiserum, according to the methods of Towbin et al. (*41*), with modifications (*37*), followed by 5×10^5 cpm/mL ^{125}I protein A (PRI/FCRF). Filters were exposed overnight to Kodak XAR film and resulting autoradiograms were scanned in an LKB Ultroscan XL Densitometer. The representative experiment in Figure 3 shows results from densitometric analysis of P^+ and P^- cells treated with TPA over a 24-hr time course. Data are plotted as relative optical intensity, using a value of 1.0 for the TPA-untreated 0 time control in the P^+ Cl 41 cells.

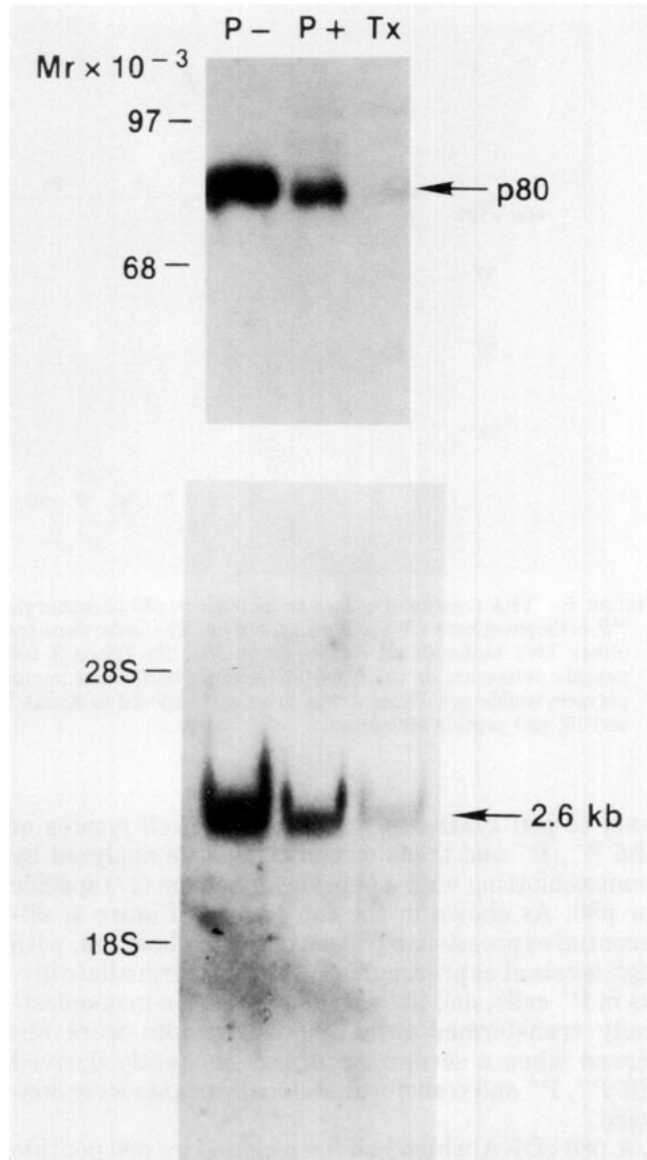

FIGURE 4. Differential expression of p80 mRNA hybridizing to a putative p80 clone from JB6 cells. (Top panel) Mouse JB6 cell clone 30 (P^-), 41 (P^+), or RT101 (Tx) were lysed in Laemmli buffer (*40*) and loaded onto an 10% SDS polyacrylamide gel. The gel was immunoblotted with p80 peptide antiserum provided by D. Kligman (*47*) (diluted 1:500) as described in Figure 3. Each lane contained an equivalent amount of cellular protein (20 μg). (Bottom panel) P^-, P^+, and transformed total cellular RNAs were isolated according to the procedure of Deeley et al. (*51*) and subjected to Northern analysis as described in Simek et al. (*20*). Each lane contained 10 μg of total cellular RNA. The filters were exposed to Kodak XAR film for 2 days.

tion and possibly neoplastic transformation. Since transactivation responses appear to persist beyond the time course of Jun induction it is possible that, while a Jun threshold may be necessary, it may not be sufficient for transactivation or promotion in this system. The precise role and mechanisms of control of cellular responsiveness by the AP-1 complex continue to be investigated.

Differential Expression of an 80-kDa/pI 4.5 Protein during Preneoplastic Progression

Previous investigations in this laboratory demonstrated a differential basal and induced levels of phosphorylated of p80 during preneoplastic progression in JB6 cells (*46*). Two-dimensional gel electrophoresis of proteins labeled *in vivo* with ^{32}P-orthophosphate showed high levels of a phosphorylated 80 kDa/pI 4.5 protein in P^- cells, intermediate levels in P^+ cells, and essentially none in neoplastically transformed derivatives of JB6 cells (*46*). Exposure to TPA caused elevated p80 phosphorylation in P^- and P^+ cells, but not in transformed cells. These results raised the question of whether this differential regulation was occurring at the pretranslational or posttranslational level. To determine whether the regulation was at the

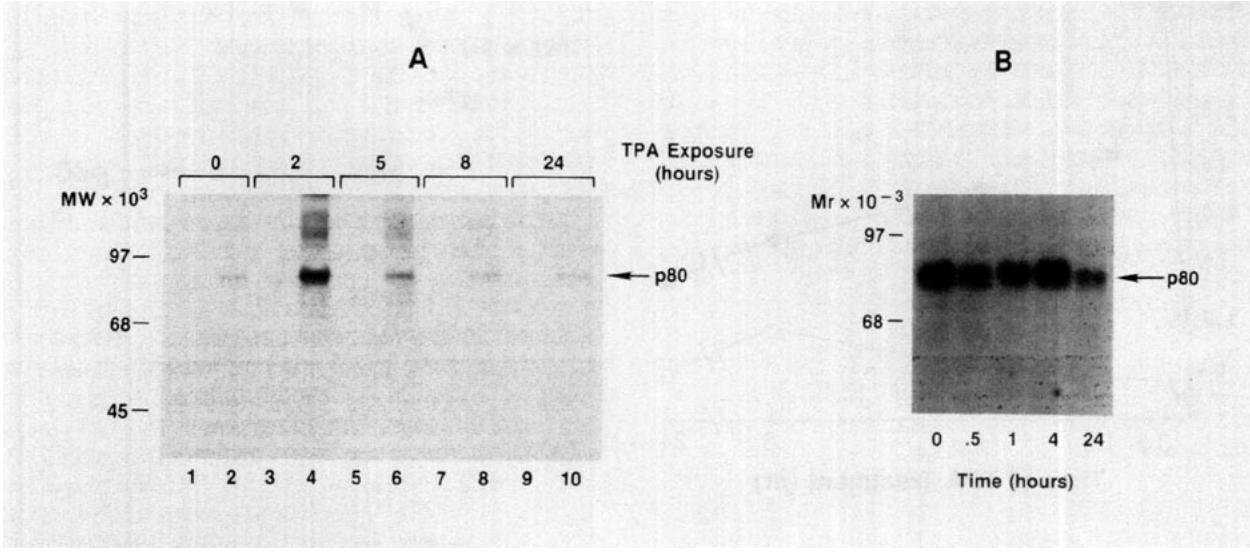

FIGURE 5. TPA treatment causes an increase in p80 phosphorylation but not in p80 synthesis. JB6 clone 30 (P^-) cells were labeled with ^{32}P orthophosphate (200 μCi/mL) for 2 hr. The cells were treated with TPA (10 ng/mL) for various times. The cells were lysed in either TNT buffer (0.2M NaCl 0.02 M Tris, 1% Triton X-100) and (*A*) immunoprecipitated with either preimmune or immune p80 peptide antiserum or (*B*) lysed in Laemmli buffer and immunoblotted as described in Figure 4. Samples were loaded onto a 10% polyacrylamide gel. Panel *A* was dried and exposed to Kodak XAR film for 24 hr. Lanes 1, 3, 5, 7, and 9 preimmune; lanes 2, 4, 6, 8, and 10, p80 peptide antiserum.

level of p80 synthesis, proteins from cell lysates of JB6 P^-, P^+ and transformed cells were analyzed by immunoblotting with a peptide antiserum (*47*) specific for p80. As shown in the top panel of Figure 4, differential expression of p80 protein was observed, with high levels of expression in P^- cells, intermediate levels in P^+ cells, and little or no expression in neoplastically transformed cells. Similar results were observed when a second set of independently derived JB6 P^-, P^+ and transformed clonal variants were analyzed.

A p80 cDNA which had been cloned by p80 peptide antibody screening (*20*) was used to analyze JB6 cellular p80 mRNA expression and to determine the extent to which p80 protein levels might be limited by p80 mRNA concentration. As shown in the bottom panel of Figure 4, when this p80 clone was used as a probe against P^- and P^+ total cellular RNA, a single 2.6-kb band was observed, but little or no hybridization was seen with RNA from transformed cells. Densitometric analysis from three experiments showed the mean value for the hybridizing band in P^+ RNA was 50 ± 2% and transformed RNA was 2.5 ± 0.4% of the P^- RNA value. This pattern was nearly identical to that observed for the differential expression of p80 protein in these cells, indicating that intracellular p80 protein concentration is regulated by the levels of p80 mRNA.

To determine whether TPA induces p80 phosphorylation and thereby test the hypothesis that p80 is a PKC substrate, TPA treated JB6 P^- cell lysates were immunoprecipitated with p80 peptide antiserum. Figure 5*A* shows the pattern of p80 phosphorylation in JB6 P^- cells treated with TPA for 0, 2, 5, 8, and 24 hr. This experiment showed an increase in p80 phosphorylation with a 6-fold maximum at 2 hr after initiation of TPA treatment (lane 4) that persisted for 5 hr (lane 6) and returned to basal levels by 24 hr (compare lanes 10 and 2). This time course was comparable to studies done in this laboratory (*13*) and by others (*48*) not using p80 antibody. The decrease in p80 phosphorylation occurred after 24 hr of TPA treatment was paralleled in JB6 cells by a decrease in protein kinase C activity and concentration (data not shown). This result correlated with findings demonstrating that treatment of cells with phorbol esters leads to progressive downmodulation of phorbol ester receptors (*49*) followed by disappearance of protein kinase C activity (*50*). Thus, phosphorylation of p80 in JB6 cells is dependent on protein kinase C, and p80 may or may not be a direct protein kinase C substrate.

To determine whether the observed increase in p80 phosphorylation reflected an increase in synthesis or was controlled posttranslationally, JB6 P^- cells were exposed to TPA for 0.5, 1, 4, and 24 hr. Cell lysates were then analyzed by immunoblotting for levels of p80, using p80 peptide antiserum. The results of this experiment are shown in Figure 5*B*. The level of p80 did not increase after tumor promoter treatment but actually appeared to decrease after prolonged TPA exposure (24 hr). Shorter TPA exposure times were also tested and again showed no increase in p80 synthesis. The results from this experiment confirmed that p80 synthesis was not increased by exposure to

TPA. In addition, total cellular RNA, isolated from P^- cells after TPA treatment for 0, 4, and 24 hr, showed no difference in the level of p80 hybridizing RNA (data not shown). Therefore, this study indicates that TPA treatment specifically induces the phosphorylation and not the synthesis of the p80 protein.

Conclusions

Regulation of c-*jun* expression in response to TPA and the subsequent activation of a specific set of target genes in response to AP-1 may play a part in the tumor promotion process. A number of phorbol ester and growth factor inducible genes have been found to contain TREs in their promoter regions, and their expression is believed to be regulated via binding of AP-1 to this promoter element. These genes include stromelysis/transin (*30,53*) and collagenase (*30,54*), proteases that may play a role in tumor invasiveness and metastasis, metallothionein II_A (*30*), and interleukin-2 (*55*). In addition, AP-1 is thought to be involved in the positive autoregulation of c-*jun* (*56*) and in both positive and negative autoregulation of c-*fos* (*57–60*). Thus, differences in tumor-promoter-induced gene activation by the AP-1 transcription factor between P^+ and P^- cells may lead to different patterns of expression of key effector genes. The defective AP1-dependent transactivation observed in P^- cells may account for their promotion-resistant phenotype. Relevant target genes for the promotion process in these cells remain to be identified.

In addition to transiently regulated genes, certain genes are constitutively switched on or switched off during preneoplastic progression (*61,62*). The 80 kDa protein observed in JB6 cells appears to be such a negatively regulated protein. The fact that p80 levels decrease as cells progress toward the neoplastic end point is compatible with its postulated role as a tumor suppressor. Whether p80 is coordinately regulated by, or with, the AP-1 protein via signals transduced by protein kinase C is currently unknown. Further investigations are underway to elucidate whether and by what mechanisms AP-1 and p80 modulate susceptibility to transformation promotion and progression to the neoplastically transformed cellular phenotype.

The authors gratefully acknowledge the excellent technical assistance of Edmund Wendel and Michael Weedon and the editorial assistance of Joyce Vincent.

REFERENCES

1. Drinkwater, N. Genetic control of hepatocarcinogenesis in inbred mice. In: Genes and Signal Transduction in Multistate Carcinogenesis (N.H. Colburn, Ed.), Marcel Dekker, New York, 1989, pp. 3–17.
2. DiGiovanni, J. Genetic determinants of susceptibility to mouse skin tumor promotion in inbred mice. In: Genes and Signal Transduction in Multistage Carcinogenesis (N.H. Colburn, Ed.), Marcel Dekker, New York, 1989, pp. 39–67.
3. Gould, M. N., Wang, B., and Moore, C. Modulation of mammary carcinogenesis by enhancer and suppressor genes. In: Genes and Signal Transduction in Multistage Carcinogenesis (N. Colburn, Ed.), Marcel Dekker, New York, 1989, pp. 19–38.
4. Malkinson, A. M. The genetic basis of susceptibility to lung tumors in mice. Toxicology 54: 241–271 (1989).
5. Colburn, N. H., Former, B. F., Nelson, K. A., and Yuspa, S. H. Tumor promoter induces anchorage independence irreversibly. Nature 281: 589–591 (1979).
6. Colburn, N. H., Vorder Bruegge, W. F., Bates, J., and Yuspa, S. H. Epidermal cell transformation *in vitro*. In: Mechanisms of Tumor Promotion and Cocarcinogenesis (T.J. Slaga, A. Sivak, and R.K. Boutwell, Eds.), Raven Press, New York, 1978, pp. 257–271.
7. Colburn, N. H., Koehler, B., and Nelson, K. A. A cell culture assay for tumor promoter dependent progression toward neoplastic phenotype: detection of tumor promoters and tumor inhibitors. Teratog. Carcinog. Mutagen. 1: 87–96 (1980).
8. Colburn, N. H., Dion, L. D., and Wendel, E. J. The role of mitogenic stimulation and specific glycoprotein changes in the mechanism of late-stage tumor promotion in JB6 epidermal cell lines. In: Carcinogenesis: A Comprehensive Survey (E. Hecker, N. E. Fusening, W. Kunz, F. Marks, and H. W. Thielmann, Eds.), Raven Press, New York, 1982, pp. 231–235.
9. Colburn, N. H., Srinivas, L., and Wendel, E. J. Responses of preneoplastic epidermal cells to tumor promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Cell Biochem. 18: 251–270 (1982).
10. Gindhart, T. D., Nakamura, Y., Stevens, L. A., Hegamyer, G. A., West, M. W., Smith, B. M., and Colburn, N. H. Genes and signal transduction in tumor promotion: conclusions from studies with promoter resistant variants of JB6 mouse epidermal cells. In: Tumor Promotion and Enhancement in the Etiology of Human and Experimental Respiratory Tract Carcinogenesis (M. Mass, Ed.), Raven Press, New York, 1985, pp. 341–368.
11. Colburn, N. H., Wendel, E., and Abruzzo, G. Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: mitogen resistant variants are sensitive to promotion. Proc. Natl. Acad. Sci. U.S.A. 78: 6912–6916 (1981).
12. Smith, B. M., Gindhart, T. D., and Colburn, N. H. Possible involvement of a lanthanide sensitive protein kinase C substrate in lanthanide promotion of neoplastic transformation. Carcinogenesis 7: 1949–1956 (1986).
13. Smith, B. M., and Colburn, N. H. Protein kinase C and its substrates in tumor promoter sensitive and resistant cells. J. Biol. Chem. 263: 6424–6431 (1988).
14. Lerman, M. I., Hegamyer, G. A., and Colburn, N. H. Cloning and characterization of putative genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. Int. J. Cancer 37: 293–302 (1986).
15. Garrity, R. R., Seed, J. L., Young, H. A., Winterstein, D., and Colburn, N. H. Evidence that mouse promotion sensitivity gene pro-1 is transcribed by RNA polymerase III. Gene 68: 63–72 (1988).
16. Srinivas, L., Gindhart, T. D., and Colburn, N. H. TPA-resistant cells lack trisialoganglioside (GT) response. Proc. Natl. Acad. Sci. U.S.A. 79: 4988–4991 (1982).
17. Nakamura, Y., Gindhart, T. D., Winterstein, D., Tomita, I., Seed, J. L., and Colburn, N. H. Early superoxide dismutase-sensitive event promotes neoplastic transformation in mouse epidermal JB6 cells. Carcinogenesis 9: 203–207 (1988).
18. Muehlematter, D., Larsson, R., and Cerutti, P. Active oxygen induced DNA strand breakage and poly ADP ribosylation in promotable and non-promotable JB6 mouse epidermal cells. Carcinogenesis 9: 239–245 (1988).
19. Bernstein, L. R., and Colburn, N. H. AP-1/*jun* function is differentially induced in promotion sensitive and resistant JB6 cells. Science 244: 566–569 (1989).
20. Simek, S. L., Kligman, D., Patel, J., and Colburn, N. H. Dif-

ferential synthesis of an 80 kDa PKC substrate in preneoplastic and neoplastic mouse JB6 cells. Proc. Natl. Acad. Sci. U.S.A. 86: 7410–7414 (1989).
21. Dion, L. D., DeLuca, L., and Colburn, N. H. Phorbol ester induced anchorage independence and its antagonism by retinoic acid correlates with altered expression of specific glycoproteins. Carcinogenesis 2: 951–958 (1981).
22. Dion, L. D., Bear, J., Bateman, J., DeLuca, L. M., and Colburn, N. H. Tumor promoting phorbol ester inhibits procollagen synthesis in promotable JB6 mouse peridermal cells. J. Natl. Cancer Inst. 69: 1147–1154 (1982).
23. Sobel, M. E., Dion, L. D., Vuust, J., and Colburn, N. H. Tumor promoting phorbol esters inhibit procollagen synthesis at a pretranslational level in mouse epidermal JB6 cells. Mol. Cell. Biol. 3: 1527–1532 (1983).
24. Copley, M., Gindhart, T., and Colburn, N. Hexose uptake as an indicator of JB6 mouse epidermal cell resistance to the mitogenic activity of TPA. J. Cell Physiol. 114: 173–178 (1983).
25. Colburn, N. H., Ozanne, S., Lichti, U., Ben, T., Yuspa, S., Wendel, E., Jardini, E., and Abruzzo, G. Retinoids inhibit promoter-dependent preneoplastic progression in mouse epidermal cell lines. Ann. N.Y. Acad. Sci. 359: 251–259 (1981).
26. Garrity, R. R., Smith, B. M., and Colburn, N. H. Genes and signals involved in tumor promoter induced transformation. In: Genes and Signal Transduction in Multistage Carcinogenesis (N.H. Colburn, Ed.), Marcel Dekker, New York, 1989, pp. 139–166.
27. Srinivas, L., and Colburn, N. H. Tumor promoter induced ganglioside changes in promotable mouse JB6 cells: antagonism by an antipromoter. J. Natl. Cancer Inst. 68: 469–473 (1982).
28. Lerman, M. I., and Colburn, N. H. Pro genes, a novel class of genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters. In: Tumor Promoters: Biological Approaches for Mechanistic Studies and Assay Systems (R. Langenbach, Ed.), Raven Press, New York, 1988, pp. 357–385.
29. Carter, T. H. The regulation of gene expression by tumor promoters. In: Mechanisms of Environmental Carcinogenesis, Vol. I (J.C. Barrett, Ed.), CRC Press, Boca Raton, FL, 1987, pp. 47–80.
30. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell 49: 729–39 (1987).
31. Lee, W., Mitchell, P., and Tjian, R. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49: 741–752 (1987b).
32. Rauscher, F. J., III, Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tjian, R., and Franza, B. R., Jr. Fos-associated protein p39 is the product of the *jun* oncogene. Science 240: 1010–1016 (1987).
33. Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, I. M. Fos associated cellular p39 is related to nuclear transcription factor AP-1. Cell 54: 553–560 (1988b).
34. Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. The c-fos protein interacts with c-*jun*/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54: 541–552 (1988a).
35. Nekabeppu, Y., Ryder, K., and Nathans, D. DNA binding activities of three murine Jun proteins: stimulation by fos. Cell 55: 907–915 (1988).
36. Schutte, J., Minna, J. D., and Birrer, M. J. Deregulated expression of human c-*jun* transforms primary rat embryo cells in cooperation with an activated c-Ha-*ras* gene and transforms Rat-1a cells as a single gene. Proc. Natl. Acad. Sci. U.S.A. 86: 2257–2261 (1989).
37. Bernstein, L. R. AP-1/*jun* is Differentially Regulated in Promotion Sensitive and Resistant Mouse Epidermal JB6 Cells. Ph.D. Thesis Dissertation, The Johns Hopkins University, Baltimore, MD, 1989.
38. Bohmann, D., Bos, T. J., Admon, A., Nishimuna, T., Vogt, P. K., and Tjian, R. Human proto-oncogene c-*jun* encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 238: 1386–1392 (1987).
39. Bos, T. J., Bohmann, D., Tsuchie, H., Tjian, R., and Vogt, P. K. v-*jun* encodes a nuclear protein with enhancer binding properties of AP-1. Cell 52: 705–712 (1988).
40. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–85 (1970).
41. Towbin, H., Staehelin, T., and Gordon, G. J. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheet: procedure and some simple applications. Proc. Natl. Acad. Sci. U.S.A. 76: 4350–4354 (1979).
42. Lamph, W. W., Wamsley, P., Sassone-Corsi, P., and Verma, I. Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature 334: 629–631 (1988).
43. Brenner, D. A., O'Hara, M., Angel, P., Chojkier, M., and Karin, M. Prolonged activation of *jun* and collagenase genes by tumor necrosis factor alpha. Nature 337: 661–663 (1989).
44. Greenberg, M. E., and Ziff, E. B. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature 311: 433–438 (1984).
45. Ben-Ari, E. T., and Garrison, J. C. Regulation of angiotensinogen mRNA accumulation in rat hepatocytes. Am. J. Physiol. 255 (Endocrinol. Metab. 18): E70–E79 (1988).
46. Gindhart, T. D., Stevens, L., and Copley, M. P. Transformation and tumor promoter sensitive phosphoproteins in JB-6 mouse epidermal cells: one is also sensitive to heat stress. Carcinogenesis 5: 1115–1121 (1984).
47. Hornbeck, P., Nakabayashi, H., Fowlkes, B. J., Paul, W. E. and Kligman, D. Protein kinase C and a major myristylated substrate of protein kinase C are differentially regulated during murine B and T lymphocyte development and activation. Mol. Cell. Biol. 9: 3727–3735 (1989).
48. Rodriguez-Pena, A., and Rozengurt, E. Phosphorylation of an acidic mol. wt. 80,000 cellular protein in a cell-free system and intact Swiss 3T3 cells: a specific marker of protein kinase C. EMBO J. 5: 77–83 (1986).
49. Collins, M. K., and Rozengurt, E. Homologous and heterologous mitogenic desensitization of Swiss 3T3 cells to phorbol esters and vasopressin: role of receptor and post-receptor events. J. Cell. Physiol. 118: 133–142 (1984).
50. Ballester, R., and Rosen, O. R. Fate of immunoprecipitable protein kinase C in GH_3 cells treated with phorbol 12-myristate 13-acetate. J. Biol. Chem. 260: 15194–15199 (1985).
51. Deeley, R. G., Gordon, J. I., Burns, A. T. H., Mullinix, K. P., Binastein, M., and Goldberger, R. F. Primary activation of vitellogenin gene in rooster. J. Biol. Chem. 252: 8310–8312 (1977).
52. Thomas, P. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77: 5201–5205 (1980).
53. Kerr, L. D., Holt, J. T., and Matrisian, L. M. Growth factors regulate transin gene expression by c-*fos*-dependent and c-*fos* independent pathways. Science 242: 1424–1427 (1988).
54. Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H. J., and Herrlich, P. 12-*O*-Tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. Mol. Cell. Biol. 7: 2256–2266 (1987).
55. Muegge, K., Williams, T. M., Kant, J., Karin, M., Chiu, R., Schmidt, A., Siebenlist, U., Young, H. A., and Durum, S. K. Interleukin-1 costimulatory activity on the interleukin-2 promoter via AP-1. Science 246: 259–251 (1989).
56. Angel, P., Hattori, K., Smeal, T., and Karin, M. The *jun* proto-oncogene is positively autoregulated by its product, jun/AP-1. Cell 55: 875–885 (1988).
57. Fisch, T. M., Prywes, R., and Roeder, R. G. An AP1-binding site in the c-*fos* gene can mediate induction by epidermal growth factor and 12-*O*-tetradecanoyl phorbol 13-acetate. Mol. Cell. Biol. 9: 1327–1331 (1989).
58. Sassone-Corsi, P., Sisson, J. C., and Verma, I. M. Transcriptional autoregulation of the proto-oncogene *fos*. Nature 334: 314–319 (1988).
59. Konig, H., Ponta, H., Rahmsdorf, U., Buscher, M., Schonthal,

A., Rahmsdorf, H. J., and Herrlich, P. Autoregulation of *fos*: the dyad symmetry element as the major target of repression. EMBO J. 8: 2559–2566 (1989).
60. Shaw, P. E., Frasch, S., and Nordheim, A. Repression of *c-fos* transcription is mediated through $p67^{SRF}$ bound to the SRE. EMBO J. 8: 2567–2574 (1989).
61. Koi, M., and Barrett, J. C. Loss of tumor suppressor function during chemically induced neoplastic progression of Syrian hamster embryo cells. Proc. Natl. Acad. Sci. U.S.A. 83: 5292–5296 (1986).
62. Stanbridge, E. J. Identifying tumor suppressor genes in human acolorectal cancer. Science 247: 12–13 (1990).